Microbiology of the association

J. Balandreau
Centre de Pédologie Biologique du Centre National de la Recherche Scientifique, B.P. 5, 54501 Vandoeuvre-les-Nancy Cédex

Accepted March 14, 1983

Counting and isolating rhizosphere N_2-fixing bacteria 851
The starting material and expression of results 852
The criterion for nitrogenase 852
The selective medium 852
Giving a name 853
Taxonomy 854
Interstrain differences 855
Conclusion 855

For personal use only.

Almost a century ago, Beijerinck (1888) isolated the first N_2-fixing bacterium, which was a *Rhizobium*. Very shortly after, Winogradsky (1893) established that *Clostridium pasteurianum* could fix atmospheric N_2 and Beijerinck (1901) isolated and described the first *Azotobacter*. In 1889, Frank had already suspected N_2 fixation to occur among *Cyanobacteria*. Thus, within a few years, was established what has remained for a very long time the basis of N_2-fixing bacteria taxonomy: free-living aerobic heterotrophic bacteria, *Azotobacteraceae*; free-living facultative and strict anaerobic heterotrophic bacteria, *Bacillaceae*; symbiotic bacteria, *Rhizobiaceae*; and free-living autotrophic bacteria, *Cyanobacteria*.

This early taxonomy was based upon ecological and physiological features and was used without significant alteration until recently. The only major change was an increase of the number of taxa falling into each category: *Azotobacteraceae* (Pribram 1933) progressively included *Azomonas* (Winogradsky 1938), *Beijerinckia* (Derx 1950), and *Derxia* (Jensen *et al.* 1960); N_2-fixing *Bacillaceae* were shown to include *Bacillus* species (Hino and Wilson 1958), and a number of N_2-fixing *Cyanophyceae* were evidenced as well as phototrophic sulfur and nonsulfur bacteria (Lindstrom *et al.* 1949).

Reports in the literature appeared also about N_2 fixation by *Pseudomonas*, *Achromobacter*, Coryneforms, etc. Many of these reports were considered to be controversial (Hill and Postgate 1969) but asked the interesting question of the pattern of distribution of the ability to fix N_2 and its relationship with taxonomy and phylogeny.

More recently, several important additions were progressively made to this classification, especially *Azospirillum*, first described as a N_2-fixing *Spirillum* or *Vibrio* by Becking (1963), and the first N_2-fixing *Enterobacteriaceae*, described by Jensen (1958).

Obviously N_2 fixation was not restricted to *Azotobacteraceae*, *Bacillaceae*, and phototrophic bacteria.

Historically, as new bacterial types appeared able to fix N_2, new isolation procedures had to be designed. The consequence is a somewhat confusing situation as to the nature of the actual bacteria responsible for nitrogenase activities associated with nonleguminous roots. Each laboratory uses a particular technique for counting and isolating N_2-fixing bacteria. The technique used dictates the type of bacterium found. Consequently each laboratory has a tendency to consider that "its own" bacterium has a prominent role.

At the very moment when confusion is maximum about the taxonomy, relative abundances, and ecological roles of many N_2-fixing bacterial species, there is an urgent need to better characterize the degree of heterogeneity of those species. The early inoculation experiments have shown that strains are not equivalent, but very little is known about the nature of differences between them. There is no way to predict the behaviour of a strain to be used as an inoculum; the only means to evaluate its potential is to try it in the field. It is unacceptable to continue to work with no directive pattern when comparing strains, and it is urgent to further characterize strain-to-strain differences and relate them to the bacterial potential.

This article is not an exhaustive review of existing literature; it is rather intended as a discussion of the impact of recent findings upon the above-mentioned problems; it centres on (*i*) methodology in use for counting and isolating rhizosphere N_2-fixing bacteria, (*ii*) identification and (*iii*) taxonomy of some N_2-fixing bacteria, and finally (*iv*) interstrain differences.

Counting and isolating rhizosphere N_2-fixing bacteria

Counts of N_2-fixing bacteria are usually done on a selective medium by characterizing the presence of nitrogenase in dilutions of the initial sample. Isolations can be made using the highest dilutions exhibiting nitrogenase activity. Problems are encountered at three different levels: (*i*) the starting material and the expression of results, (*ii*) the criterion for nitrogenase, and (*iii*) the selective medium.

The starting material and expression of results

There is no functional definition of the rhizosphere soil; it is most often defined as the soil adhering to the roots after "gentle" shaking. This definition depends upon what is considered to be "gentle." It is possible that the total number of bacteria recovered does not depend very much upon the extent of shaking because most of the bacteria adhere very firmly to the root surface. However, the question still remains important because the results are often expressed per unit weight of soil, making it difficult to compare results published by different workers. Expressing the results per unit weight of root is not an improvement, given the variety of root types found on the same plant species depending on age, distance from the crown, etc. Thus, the only unequivocal way to obtain comparable results would be to calculate numbers of bacteria on a per plant basis, taking into account the total per plant mass of roots or of rhizosphere soil, whatever its definition. To make allowance for plant size, perhaps it would be better still to consider the size of its photosynthetic apparatus and express the numbers of rhizosphere N_2-fixing bacteria per gram dry weight of green leaves. An advantage would thus be to better characterize the relationship between the potentials for C and N fixation, an important feature to compare among N_2-fixing associations. Finally, this expression would hopefully permit an easier comparison of counts made by different people on plants differing by their species, age, size, and location.

The criterion for nitrogenase

It is generally agreed (Hill and Postgate 1969) that growth on a nitrogen-free medium is not a sufficient criterion for nitrogenase and the more so as some N_2-fixing bacteria cannot grow on a medium completely free of combined nitrogen (Watanabe and Barraquio 1979).

As a measure of N_2 fixation ^{15}N incorporation is often too expensive to be used on a large scale, and many authors have used C_2H_2 reduction as an indirect assay technique. In counting experiments this technique can present some difficulties owing to the shortness of the period during which C_2H_2 reduction can be evidenced and to C_2H_4 oxidation by some bacteria.

Many N_2-fixing bacteria appear to express nitrogenase activity only when they are in sufficient numbers (Haucke-Pacewiczowa *et al.* 1970; Brouzes *et al.* 1971). In enumeration experiments, this threshold is reached only after a lag period that is dependent upon the initial population of the inoculum (Villemin *et al.* 1974). With high dilutions this can take as long as 3 weeks. Moreover once established, nitrogenase activity can be rapidly suppressed by carbon or oxygen limitation so that the overall period of time during which nitrogenase activity occurs can be very short. This difficulty may be overcome by waiting long enough for each tube containing N_2-fixing bacteria to develop a sufficiently large population and then to add fresh medium and incubate under C_2H_2. Usually a very high level of activity can be obtained within 2 days (Villemin *et al.* 1974). Another possibility is to incubate the replicate tubes under 1% C_2H_2 as soon as they are inoculated. This permits sufficient N_2 fixation for the growth of large populations in all positive tubes and is enough to produce detectable amounts of C_2H_4 wherever there is nitrogenase activity.

Ethylene oxidation can be another problem in counting experiments in which C_2H_2 reduction is used as a criterion for nitrogenase. Ethylene oxidation has been shown by Yoshida (Yoshida 1970; Yoshida and Suzuki 1975) but is generally overlooked, except in the studies of de Bont (de Bont and Mulder 1974, 1976; de Bont 1976; de Bont and Albers 1976; de Bont and Harder 1978; de Bont *et al.* 1980). Two types of artifacts could occur: C_2H_4 evolution, rapidly followed by C_2H_4 consumption (Yoshida 1970), or inhibition of growth of CH_4-utilizing N_2-fixing bacteria (de Bont and Mulder 1976). Both phenomena are aerobic, the former has been evidenced in rice fields and the latter is likely to occur in waterlogged soils on a small scale (de Bont *et al.* 1978). Their incidence on enumeration of N_2-fixing bacteria does not seem to have been thoroughly investigated.

The selective medium

The nature of the carbon source used in N-free media encourages the growth of different groups of bacteria in counting and in isolating procedures. For instance, use of mannitol or glucose leads to the enumeration and isolation of *Azotobacteraceae* (Thompson and Skermann 1979) and use of malate to the isolation of *Azospirillum* (Okon *et al.* 1977). As far as rhizosphere bacteria are concerned, it would seem advisable to use C sources more similar to those used *in situ* by the bacteria, i.e., the root exudates. Several procedures can be considered such as the use of axenically collected exudates (Rubenchik 1963) or the use of media containing a mixture of carbon sources (Rennie 1981); however, the "spermosphere model" (Thomas-Bauzon *et al.* 1982) seems most promising, in which use is made of a young sterile seedling growing on a mineral medium free of C and N. The source of energy and C for N_2-fixing bacteria is constituted by the exudates. It is assumed that they have a composition similar enough to those of an older plant to introduce no C bias. The spermosphere tubes are inoculated with rhizosphere soil dilutions and closed by Suba-seal rubber stoppers, 1% C_2H_2 is injected, and

C_2H_4 is measured after incubation for 1 week. The calculation of the most probable number of N_2-fixing bacteria in the original sample is based upon the number and distribution of tubes containing C_2H_4. The contents of the tubes of the highest dilution in which all the tubes are C_2H_4 positive can then be used as a starting material for further isolation of N_2-fixing bacteria. The authors have shown that the percentage of N_2-fixing bacteria is very high in the spermosphere model (65% in their example) compared with the percentage in the initial soil (6.6%). This is not so in more regular counting procedures using artificial C sources; in nitrogenase positive tubes, N_2-fixing bacteria are often readily replaced by nonfixing bacteria that use any nitrogen made available by the former, so increasing the difficulty of using C_2H_4-positive tubes for isolation of N_2-fixing microorganisms. In the spermosphere model, it seems that the plantlet may help keep the medium selective by scavenging any available nitrogen. This spermosphere model has been used with rice and maize with equal success.

In many instances N_2 fixing bacteria have been isolated from actively C_2H_2-reducing root pieces incubated at low oxygen tension in a malate medium. This method (Döbereiner and Day 1976) is very efficient for isolating *Azospirillum* from active roots but does not warrant the conclusion that isolated strains are representative of the N_2-fixing microflora of the undisturbed plant. There is often a long delay between sampling time and the start of incubation. During this delay roots probably exhaust their share of assimilates and the normal flux of exudates is likely to stop. Moreover, there appears to be a large and rapid increase in number of N_2-fixing bacteria (up to 30 times) within 16 h (Barber *et al.* 1976; Koch 1977). It seems very probable that carbon and (or) energy necessary to this multiplication and subsequent C_2H_2-reducing activity are not only derived from exudates but also from root fermentation products, or perhaps ethanol introduced with bromothymol blue. It would be interesting to evaluate the percentage of ethanol-utilizing *Azospirillum* strains isolated following this procedure. Thus, it is probable that this method introduces artifacts by selectively stimulating bacteria adapted to low redox potentials and root fermentation.

Giving a name

There have been several recent reviews about N_2-fixing bacterial genera and species (Mulder and Brotonegoro 1974; Knowles 1977, 1978; La Rue 1977; Postgate 1981); the reader should refer to them for general consensus about "classical" nitrogen fixers. The present article will deal very briefly with genera easy to identify and will focus on some generally underestimated problems about rhizosphere N_2-fixing bacteria.

First of all one must comment on the fact that many soil microbiologists are not very keen on taxonomy. They publish results concerning strains easily identified such as *Bacillus*, *Clostridium*, *Enterobacteriaceae*, "*Azotobacteraceae*," and *Azospirillum*, but rarely about other isolates. Nevertheless, most of them, privately, admit that they have often isolated unknown bacteria. Not only are descriptions of such isolates very rarely given, but in most cases strains are not maintained and thus are not available for subsequent precise characterization by other laboratories.

Microorganisms belonging to the *Bacillus* and *Clostridium* genera are easily identified and little needs to be said about them, except that the ecology of the former should be further investigated (Abdel Wahab 1975; Nelson *et al.* 1976; Neal and Larson 1976; Jordan *et al.* 1978).

N_2-fixing *Enterobacteriaceae* are very common and rather easy to identify as short gram-negative rods, catalase positive, oxidase negative, growing on McConkey agar. Their precise determination may be facilitated by the use of API 20 E microtube cards (Rennie 1980).

"*Azotobacteraceae*" have been abundantly described (Thompson and Skermann 1979); nevertheless, new rhizosphere-adapted species, similar to "*Azotobacter*" *paspali* will possibly be discovered. Moreover, occurrence of *Beijerinckia* in nontropical rhizospheres has been underestimated and may prove more common than previously thought (Jordan *et al.* 1978).

With regard to *Azospirillum*, several problems arise. The characterization of the genus *Azospirillum* includes the special type of motility as a criterion. The recent isolation of a nonmotile strain of *Azospirillum* in our laboratory indicates the need for a reevaluation of this generic feature (Bally *et al.* 1983). Again, at the species level, definitive characteristics are not always very clear. Acidification of glucose media is not specific of *A. lipoferum*; among *A. brasilense* some strains can acidify glucose (Tarrand *et al.* 1978), and conversely some strains of *A. lipoferum* can lose this property (unpublished result). Moreover, the need for biotin can be reproducibly evidenced only after several transfers on a biotin-free medium. Those findings, along with the low degree of DNA/DNA homology (Tarrand *et al.* 1978) make difficult the distinction of two different species in *Azospirillum*.

Beside those well-known taxa, occasional reports point to the possibility of N_2 fixation in species of the following genera: *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Campylobacter*, *Pseudomonas*, *Rhizobium*, and *Xanthobacter*.

N_2 fixation by *Achromobacter* was suggested by Jensen (1958) and further studied by Proctor and Wilson (1959). The strain studied by Jensen was probably a *Klebsiella* (Mahl *et al.* 1965), but the status of other strains studied by Proctor and Wilson remains uncertain.

Watanabe and Barraquio (1979) also isolated strains first designated as *Achromobacter* (then as *Pseudomonas*-like bacteria). In fact, *Achromobacter* must be considered as a *nomen dubium* (Hendrie *et al*. 1974) and *Achromobacter pestifer* has been recently shown to belong to the *Alcaligenes* genus (Kiredjian *et al*. 1981).

Alcaligenes latus (Palleroni and Palleroni 1978) was found to actively fix N_2 by Malik *et al*. (1981); fixation occurs under low oxygen partial pressure along with hydrogen oxidation. In their recent review, Kiredjian *et al*. (1981) proposed to reject this species from the genus *Alcaligenes*. Thus, N_2 fixation by true *Alcaligenes* is still to be demonstrated.

N_2 fixation by *Arthrobacter* was reported first by Smyk and Ettlinger (1963) and seems rather widespread (Cacciari *et al*. 1979), in spite of an adverse opinion of Mulder and Brotonegoro (1974).

Recently McClung and Patriquin (1980) isolated a N_2-fixing *Campylobacter* from the rhizosphere of *Spartina alterniflora*. Usually this genus is considered to belong to the *Spirillaceae* (Krieg and Smibert 1974).

Nitrogen fixation in *Mycobacterium flavum* was demonstrated by Fedorov and Kalininskaya (1961) and studied in detail by Biggins and Postgate (1969, 1971*a*). Other apparently Gram-positive hydrogen bacteria were shown able to fix N_2, especially several strains of *Corynebacterium autotrophicum* (Gogotov and Schlegel 1974). More recently Wiegel *et al*. (1978) demonstrated that all those bacteria were not Gram positive; apparent Gram-positive staining was due to polyphosphate granules. This fact lead these authors to propose the creation of a new genus, *Xanthobacter*, to include *Corynebacterium autotrophicum* and *Mycobacterium flavum*. The authors suggest that this genus belongs to the family *Azotobacteraceae* and is distinguished from other members of this family by characters such as autotrophic growth under hydrogen, absence of growth on most sugars except fructose, growth on methanol, and the production of a characteristic carotenoid pigment, zeaxanthine dirhamnoside.

Several *Pseudomonas* isolates have been reported to fix nitrogen (Anderson 1955; Proctor and Wilson 1958; Paul and Newton 1961) and were assigned species names like *Ps. azotogensis* and *Ps. azotocolligans*. In 1966 De Ley and Park included some of those strains in a study of molecular biological taxonomy of free-living diazotrophs and concluded that they are not pseudomonads. Under reexamination by Hill and Postgate (1969), strains of both species appeared unable to fix N_2 or to reduce C_2H_2. Only the strains isolated by Voets and Debacher (1956) as *Ps. azotogensis* exhibited C_2H_2 reduction, $^{15}N_2$ fixation occurred only under anaerobiosis, and again the DNA base composition fell outside the normal range of *Pseudomonas* (De Ley and Park 1966). Those putative N_2-fixing pseudomonads have been called "ghosts" by Postgate (1981). One must also mention reports of isolation of N_2-fixing *Pseudomonas* in the rhizosphere of *Pennisetum americanum* (Prabha *et al*. 1978), maize (Berlier and Lespinat 1978), and rice (Watanabe and Barraquio 1979), but the descriptions of the strains are not sufficient to judge the validity of their assignments to the *Pseudomonas* genus.

More precise is the description of *Lignobacter* strain K 17 studied for its lignin-degradation ability by Salkinoja-Salonen *et al*. (1979) and which proved able to fix N_2. By DNA/RNA hybridization de Smedt and De Ley (1977) have shown that it was a very close relative of *Pseudomonas*. An interesting feature of this strain is that it possesses plasmid encoded *nif* genes (Deryto *et al*. 1981). It would be important to compare this strain with "ghost" N_2-fixing *Pseudomonas* studied by Anderson (1955) and Paul and Newton (1961). If there is any similarity, one could speculate that the loss of the ability to fix N_2 could be due to the loss of *nif* plasmids during prolonged storage.

In our laboratory (Thomas-Bauzon *et al*. 1982), using the spermosphere model described above, we have isolated three strains of N_2-fixing *Pseudomonas*-like bacteria from the rhizosphere of rice. They were similar to *Pseudomonas paucimobilis* (Holmes *et al*. 1977) and seemed to confirm the possibility of N_2 fixation by *Pseudomonas*. Nevertheless, further studies of those strains in De Ley's laboratory have revealed that they are closely related to *Flavobacterium capsulatum* (Bally *et al*. 1983). They seem to be the first clearly identified N_2-fixing *Flavobacterium*-like strains; the only previous claim for N_2 fixation by *Flavobacterium* is by Yoshida (1970).

At the end of this survey of difficulties encountered when giving a name to some nonclassical N_2-fixing bacteria, it must be clearly said that beside easily identified groups (*Bacillus*, *Clostridium*, *Azotobacter*, *Enterobacteriaceae*, and *Azospirillum*) there may be a large number of underinvestigated N_2-fixing species, genera, or even higher taxonomic entities (Actinomycetes, for instance). We must consider that we are far from knowing all of the N_2-fixing soil bacteria that exist and should continue to put our efforts into more precise descriptions and taxonomic studies of the bacteria with which we are working.

Taxonomy

Traditional taxonomy has given a great weight to N_2 fixation as a taxonomic character. At least two families were based upon this character (*Azotobacteraceae* and *Rhizobiaceae*). The more we learn about N_2-fixing bacteria the more we see that, in fact, N_2 fixation has probably very little phylogenetic meaning.

Recent work from De Ley's laboratory (de Smedt and De Ley 1977; de Smedt *et al*. 1980) has brought about a very important modification in Gram-negative bacteria taxonomy, based upon a study of similarities of ribosomal ribonucleic acid cistrons. The most important changes affect the taxonomy of N_2-fixing bacteria. If there are no N_2-fixing genera in group V (*Cytophaga* and low (G + C)% *Flavobacterium*), all other four groups defined by these authors contain N_2-fixing genera or species.

The taxonomy of Gram-negative bacteria according to de Smedt and De Ley (1977, 1980) is as follows (names of genera and families containing N_2-fixing species are followed by an asterisk).

Group I

Vibrionaceae; *Enterobacteriaceae**; some *Alteromonas*.

Group II

Xanthomonas; marine *Alcaligenes*; fluorescent pseudomonads; some *Alteromonas*; *Azotobacter**; *Azorhizophilus** (Thompson and Skerman 1979); *Azomonas**; *Azomonotrichon** (Thompson and Skerman 1979); *Lignobacter**.

Group III

Janthinobacterium; *Chromobacterium*; *Bordetella*; most *Alcaligenes*; *Pseudomonas* species of the *acidovorans* and *solanacearum* sections; *Derxia**; the N_2-fixing *Alcaligenes latus** (Malik and Schlegel 1980; Malik *et al.* 1981) is perhaps a member of this group.

Group IV

*Rhizobium**; *Bradyrhizobium** (Jordan 1982); *Agrobacterium*; *Beijerinckia**; *Gluconobacter*; *Acetobacter*; *Pseudomonas paucimobilis** (Thomas-Bauzon *et al.* 1982); *Azotomonas*; *Aquaspirillum**; *Azospirillum**; *Rhodospirillum**; *Rhodopseudomonas**; *Paracoccus denitrificans*; *Phyllobacterium*; *Xanthobacter**.

N_2 fixation appears with a very high frequency in Group IV. It must be stressed that some misnamed *Achromobacter*, along with *Phyllobacterium* and *Azotomonas*, long suspected to fix N_2, are also located in this group. It must also be stressed that N_2 fixation has not been investigated in genera like *Zymomonas*, *Gluconobacter*, and *Acetobacter*, but the ecology of those bacteria does not make it unlikely.

This group IV also has a very interesting feature; most of its members share a common ecology, they live on plants. Some of them have even achieved the most sophisticated adaptations to coexistence with a plant: the root nodule of *Rhizobium* and the genetic parasitism of Crown-gall *Agrobacterium*. This group probably represents a bacterial phylum which has undergone a coevolution with the higher plants, from algae-associated *Aquaspirillum* to fruit-associated *Gluconobacter*.

Interstrain differences

Among N_2-fixing strains, strain-to-strain differences exist in a number of characters such as production of plant growth substances (Hennequin and Blachere 1966; Brown 1972; Gaskins *et al.* 1977; Reynders and Vlassak 1979); denitrification and resistance to antibiotics (Neyra *et al.* 1977; Dobereiner and Baldani 1979; Zuberer and Roth 1982); chemolithotrophic growth (Malik and Schlegel 1981); use of different substrates, e.g., simple sugars (Tarrand *et al.* 1978), pectic compounds (Monzon de Asconegui and Kaiser 1972; Tien *et al.* 1981), phenolic compounds (Hardisson *et al.* 1969; Hussein *et al.* 1976; Laborde and Gibson 1977; Salkinova-Salonen *et al.* 1979; Durham and Ornston 1980); antigenic properties (De Polli *et al.* 1980); and lysogenic phages (Franche and Elmerich 1981).

Moreover, many strains of N_2-fixing bacteria contain plasmids. They have been demonstrated to occur in *Rhizobium* (Casse *et al.* 1979); *Beijerinckia* (Laborde and Gibson 1977); *Xanthobacter* (Wilke 1980); *Pseudomonas paucimobilis* (Heulin *et al.* 1982); *Lignobacter* (Salkinoja-Salonen *et al.* 1979); *Enterobacter* (unpublished result); and *Azospirillum* (Franche and Elmerich 1981; Wood *et al.* 1982; Singh and Wenzel 1982; Heulin *et al.* 1982). Plasmid-encoded characters are very important to differentiate from chromosome-linked ones; only the latter constant characteristics have taxonomic value.

The presence of *nif* genes on plasmids has been demonstrated only in *Rhizobium* and *Lignobacter*, but the location on plasmids of some *Azospirillum nif* genes would probably account for the difficulty to obtain Nif^- mutants in this bacterium. The frequent loss of N_2 fixation ability during isolation procedure can only partially be accounted for by transfer to conditions nonconducive to N_2 fixation. We cannot rule out the possibility that some isolates lose N_2 fixation ability through the loss of plasmids. Postgate (1981) even speculates that *nif* is a transposable element at present spreading to new bacteria "wherever selection pressure coincides with an appropriate physiological background."

The same reasoning would lead to an even more likely hypothesis. If we take into account the frequency of plasmids in plant-associated bacteria, can we not speculate that some of those plasmids code for adaptive traits conferring to their hosts a better fitness to plant rhizospheres?

In view of the use of free-living, N_2-fixing bacteria for cereal inoculations, it seems urgent to know more about variable characters, interstrain differences, and especially the plasmid-encoded ones. It will allow us to understand more fully the mechanisms of plant–microbe coadaptations and to provide a better matching of the pro- and eu-karyotic partners in N_2-fixing associations.

Conclusion

Following this brief survey of recent developments and of some important questions in the microbiology of nonsymbiotic N_2-fixing associations, it appears that

For personal use only.

before harnessing the process we have to increase our knowledge in all the areas of its ecology, both synecology and autecology.

Our first task must be a better description of the existing N_2-fixing microflora by the use of more efficient enumeration and isolation procedures, ensuring that strains studied are effectively abundant, representative, and active. A better description of N_2-fixing microflora requires a far better identification of isolates, using modern methodologies and collection of well-identified reference strains. For instance, there are several unclear areas to investigate urgently, such as the relationships between De Ley's group IV genera of N_2-fixing bacteria, or the relationships between *Azotobacter* and N_2-fixing *Pseudomonas*-like bacteria. In the same context, it seems evident that too much consideration has been given to N_2 fixation as a characteristic in classical taxonomies. For instance, it would be very unlikely that a nonfixing *Rhizobium* would be identified, in spite of its possible great ecological significance. We need to reevaluate taxa such as *Rhizobium*, *Azotobacter*, *Beijerinckia* , and *Derxia* on grounds other than their N_2 fixation ability. We need also to investigate the possible role of several groups such as N_2-fixing actinomycetes, hydrogen bacteria, and methane bacteria.

Autecology of N_2-fixing bacteria associated with higher plants is a very young science. The following questions need answers: What would be a measure of adaptation to the rhizosphere and (or) to the rhizosphere of a particular plant species? What are the mechanisms of adaptation? Are they comparable in different bacterial families, in different plant families, in different regions of the world? Is it possible to improve such an adaptation? Is it possible to confer extra selective advantages to a bacterium by manipulating those adaptation mechanisms?

All these questions open new avenues of fundamental research and the answers are prerequisites for a sound rationale in the practical harnessing of N_2 fixation by nonsymbiotic plant-associated bacteria.

ABDEL WAHAB, A. M. 1975. Nitrogen fixation by *Bacillus* strains isolated from the rhizosphere of *Ammophila arenaria*. Plant Soil, **42**: 703–708.

ANDERSON, G. R. 1955. Nitrogen fixation by *Pseudomonas*-like soil bacteria. J. Bacteriol. **70**: 129–133.

BALLY, R., D. THOMAS-BAUZON, TH. HEULIN, J. BALANDREAU, C. RICHARD, and J. DE LEY. 1983. Determination of the most frequent N_2-fixing bacteria in a rice rhizosphere. Can. J. Microbiol. **29**. This issue.

BARBER, L. E., J. D. TJEPKEMA, S. A. RUSSEL, and H. J. EVANS. 1976. Acetylene reduction (nitrogen fixation) associated with corn inoculated with *Spirillum*. Appl. Environ. Microbiol. **32**: 108–113.

BECKING, J. H. 1963. Fixation of molecular nitrogen by an aerobic *Vibrio* or *Spirillum*. J. Microbiol. Serol. **29**: 326.

BEIJERINCK, M. W. 1888. Die Bakterien der Papilionaceenknöllchen. Bot. Zeitung (Berlin), **46**: 726–735, 741–750, 757–771, 781–790, 797–804.

——— 1901. Ueber Oligonitrophile Mikroben. Zentralbl. Bakteriol. Parasitenk. Infectionskr. **7**: 561–582.

BERLIER, Y. M., and P. A. LESPINAT. 1978. Relationship between nitrogenase and hydrogenase activity in a rhizosphere diazotroph. FEMS Microbiol. Lett. **3**: 187–190.

BIGGINS, D. R., and J. R. POSTGATE. 1969. Nitrogen fixation by cultures and cell-free extracts of *Mycobacterium flavum* 301. J. Gen. Microbiol. **56**: 181–193.

——— 1971*a*. Nitrogen fixation by extracts of *Mycobacterium flavum* 301. Use of natural electron donors and oxygen sensitivity of cell-free preparations. Eur. J. Biochem. **19**: 408–415.

——— 1971*b*. Confusion in the taxonomy of a nitrogen-fixing bacterium currently classified as *Mycobacterium flavum* 301. J. Gen. Microbiol. **65**: 119–123.

BROUZES, R., C. I. MAYFIELD, and R. KNOWLES. 1971. Effect of oxygen partial pressure on nitrogen fixation and acetylene reduction in a sandy loam soil amended with glucose. Plant Soil, Special Volume. pp. 481–494.

BROWN, M. E. 1972. Plant growth substances produced by microorganisms of soil and rhizosphere. J. Appl. Bacteriol. **35**: 443–451.

CACCIARI, I., A. GRAPPELLI, D. LIPPI, and W. PIETROSANTI. 1980. Effect of growth rate on the production of phytohormone-like substances by an *Arthrobacter* sp. in chemostat culture. J. Gen. Microbiol. **118**: 549–552.

CASSE F., C. BOUCHER, J. S. JULLIOT, M. MICHEL, and J. DENARIE. 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113**: 229–242.

DE BONT, J. A. M. 1976. Bacterial degradation of ethylene and the acetylene reduction test. Can. J. Microbiol. **22**: 1060–1062.

DE BONT, J. A. M., and R. A. J. M. ALBERS. 1976. Microbial metabolism of ethylene. Antonie van Leeuwenhoek, **42**: 73–80.

DE BONT, J. A. M., and W. HARDER. 1978. Oxidation of ethylene by *Mycobacterium* E 20. Proc. Soc. Gen. Microbiol. **5**: 68.

DE BONT, J. A. M., K. K. LEE, and D. F. BOULDIN. 1978. Bacterial oxidation of methane in a rice paddy. Ecol. Bull. (Stockholm), **26**: 91–96.

DE BONT, J. A. M., and E. G. MULDER. 1974. Nitrogen fixation and co-oxidation of ethylene by a methane-utilizing bacterium. J. Gen. Micobiol. **83**: 113–121.

——— 1976. Invalidity of acetylene reduction assay in alkane-utilizing, nitrogen-fixing bacteria. Appl. Environ. Microbiol. **31**: 640–647.

DE BONT, J. A. M., S. B. PRIMROSE, M. D. COLLINS, and D. JONES. 1980. Chemical studies on some bacteria which utilize gaseous unsaturated hydrocarbons. J. Gen. Microbiol. **117**: 97–102.

DE LEY, J., and I. W. PARK. 1966. Molecular biological taxonomy of some free-living nitrogen-fixing bacteria. Antonie van Leeuwenhoek, **32**: 6–16.

De Polli, H., B. Ben Bohlool, and J. Döbereiner. 1980. Serological differenciation of *Azospirillum* species belonging to different host-plant specificity groups. Arch. Microbiol. **126**: 217–222.

Derito, M., M. Gtowacka, A. Skorupska, and Z. Lorkiewics. 1981. Nif plasmid from *Lignobacter*. Arch. Microbiol. **130**: 322–324.

Derx, H. G., 1950. *Beijerinckia*, a new genus of nitrogen-fixing bacteria occurring in tropical soils. Proc. K. Ned. Akad. Wet. Ser. C, **53**: 140–147.

De Smedt, J., M. Bauwens, R. Tytgat, and J. De Ley. 1980. Intra- and intergeneric similarities of ribosomal ribonucleic acid cistrons of free-living, nitrogen-fixing bacteria. Int. J. Syst. Bacteriol. **30**: 106–122.

De Smedt, J., and J. De Ley. 1977. Intra- and intergeneric similarities of *Agrobacterium* ribosomal ribonucleic acid cistrons. Int. J. Syst. Bacteriol. **27**: 222–240.

Döbereiner, J., and L. D. Baldani. 1979. Selective infection of maize roots by streptomycin-resistant *Azospirillum lipoferum* and other bacteria. Can. J. Microbiol. **25**: 1264–1269.

Döbereiner, J., and J. M. Day. 1976. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. Proceedings of the 1st International Symposium on Nitrogen Fixation. *Edited by* W. E. Newton and C. J. Nyman. Washington State University Press, Pullman. pp. 518–538.

Durham, D. R., and L. N. Ornston. 1980. Homologous structural genes and similar induction patterns in *Azotobacter* spp. and *Pseudomonas* spp. J. Bacteriol. **143**: 834–840.

Fedorov, M. V., and T. A. Kalininskaya. 1961. A new species of nitrogen-fixing *Mycobacterium* and its physiological properties. Mikrobiologiya, **30**: 7–11.

Franche, C., and C. Elmerich. 1981. Physiological properties and plasmid content of several strains of *Azospirillum brasilense* and *A. lipoferum*. Ann. Microbiol. **132A**: 3–18.

Frank, B. 1889. Ueber den esperimentellen Nachweis der Assimilation freien Stickstoffes durch erdbodenbewohnende Algen. Ber. Dtsch. Bot. Ges. **7**: 34–42.

Gaskins, M. H., M. Garcia, T. M. Tien, and D. H. Hubbell. 1977. N_2-fixation and growth substance production by *Spirillum lipoferum* and their effects on plant growth. Plant Physiol. **59**: 128.

Gogotov, J. N., and H. G. Schlegel. 1974. N_2-fixation by chemoautotrophic hydrogen bacteria. Arch. Microbiol. **97**: 359–362.

Hardisson, C., J. M. Sala-Trepat, and R. Y. Stanier. 1969. Pathway for the oxidation of aromatic compounds by *Azotobacter*. J. Gen. Microbiol. **59**: 1–11.

Hauke-Pacewiczowa, T., J. Balandreau, and Y. Dommergues, 1970. Fixation microbienne de l'azote dans un sol salin tunisien. Soil Biol. Biochem. **2**: 47–53.

Hendrie, M. S., A. J. Holding, and J. M. Shewan. 1974. Emended descriptions of the genus *Alcaligenes* and of *Alcaligenes faecalis* and proposal that the generic name *Achromobacter* be rejected: status of the named species of *Alcaligenes* and *Achromobacter*. Int. J. Syst. Bacteriol. **24**: 534–550.

Hennequin, J. R., and H. Blachere, 1966. Recherches sur la synthèse de phytohormones et de composés phénoliques par *Azotobacter* et des bactéries de la rhizosphère. Ann. Inst. Pasteur, Paris, **111**: 89–102.

Heulin, Th., R. Bally, and J. Balandreau. 1982. Isolation of a very efficient N_2-fixing bacteria from the rhizosphere of rice. *In* Azospirillum, genetics, physiology, and ecology. *Edited by* W. Klingmüller. Birkhäuser verlag. Basel. Exper. Suppl. (Basel), **42**: 92–99.

Hill, S., and J. R. Postgate. 1969. Failure of putative nitrogen-fixing bacteria to fix nitrogen. J. Gen. Microbiol. **58**: 277–285.

Hino, S., and P. W. Wilson. 1958. Nitrogen fixation by a facultative *Bacillus*. J. Bacteriol. **75**: 403–408.

Holmes, B., R. J. Owen, A. Evans, H. Malnick, and W. R. Willcox. 1977. *Pseudomonas paucimobilis*, a new species isolated from human clinical specimens, the hospital environment and other sources. Int. J. Syst. Bacteriol. **27**: 133–146.

Hussein, Y. A., M. S. Tewfic, and Y. A. Hamdi. 1976. Cleavage of aromatic acids by *Azotobacter vinelandii*. Phyton, **34**: 7–9.

Jensen, V. 1958. A new nitrogen-fixing bacterium from a Danish watercourse. Arch. Microbiol. **29**: 348–353.

Jensen, H. L., E. J. Petersen, P. K. De, and R. Bhattacharya. 1960. A new nitrogen-fixing bacterium: *Derxia gummosa* nov. gen. nov. spec. Arch. Mikrobiol. **36**: 182–195.

Jordan, D. C. 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. **32**: 136–139.

Jordan, D. C., P. J. McNicol, and M. R. Marshall. 1978. Biological nitrogen fixation in the terrestrial environment of a high Arctic ecosystem (Truelove Lowland, Devon Island, N.W.T.). Can. J. Microbiol. **24**: 643–649.

Kiredjian, M., M. Popoff, C. Coynault, M. Lefevre, and M. Lemelin. 1981. Taxonomie du genre Alcaligenes. Ann. Microbiol. Paris, **132B**: 337–374.

Knowles, R. 1977. The significance of asymbiotic dinitrogen fixation by bacteria. *In* A treatise on dinitrogen fixation: IV agronomy and ecology. *Edited by* R. W. F. Hardy and W. S. Silver. Wiley Interscience, New York. pp. 33–83.

——— 1978. Free-living bacteria. *In* Limitations and potentials for biological nitrogen fixation in the tropics. *Edited by* J. Döbereiner, R. H. Burris, A. Hollaender, A. A. Franco, C. A. Neyra, and D. B. Scott. Plenum Publishing Corp., New York, pp. 25–40.

Koch, B. L. 1977. Associative nitrogenase activity by some Hawaiian grass roots. Plant Soil, **47**: 703–706.

Krieg, N. R., and R. M. Smibert. 1974. *Spirillaceae*. *In* Bergey's manual of determinative bacteriology. 8th ed. *Edited by* Buchanan and N. E. Gibbons. Williams & Wilkins Company, Baltimore. pp. 196–216.

Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a Beijerinckia species. Appl. Environ. Microbiol. **34**: 783–790.

La Rue, T. A. 1977. The bacteria. *In* A treatise on dinitrogen fixation. Vol. III. Biology. *Edited by* R. W. F. Hardy and W. S. Silver. Wiley Interscience, New York. pp. 19–62.

Lindstrom, E. S., R. H. Burris, and P. W. Wilson. 1949. Nitrogen fixation by photosynthetic bacteria. J. Bacteriol. **58**: 313–315.

For personal use only.

LINE, M. A., and M. W. LOUTIT. 1973. Studies on non-symbiotic nitrogen fixation in New Zealand tussock-grassland soils. N.Z. J. Agric. Res. **16**: 87–94.

MAHL, M. C., P. W. WILSON, M. A. FIFE, and W. H. EWING. 1965. Nitrogen fixation by members of the tribe *Klebsielleae*. J. Bacteriol. **89**: 1482–1487.

MALIK, K. A., C. JUNG, D. CLAUS, and H. G. SCHLEGEL. 1981. Nitrogen fixation by the hydrogen-oxidizing bacterium *Alcaligenes latus*. Arch. Microbiol. **129**: 254–256.

MALIK, K. A., and H. G. SCHLEGEL. 1980. Enrichment and isolation of new nitrogen-fixing hydrogen bacteria. FEMS Microbiol. Lett. **8**: 101–104.

——— 1981. Chemolithoautotrophic growth of bacteria able to grow under N_2-fixing conditions. FEMS Microtbiol. Lett. **11**: 63–67.

MCLUNG, C. R., and D. G. PATRIQUIN, 1980. Isolation of a nitrogen-fixing *Campylobacter* species from the roots of *Spartina alterniflora* Loisel. Can. J. Microbiol. **26**: 881–886.

MONZON DE ASCONEGUI, M. A., and P. KAISER. 1972. L'utilisation des produits de décomposition de la pectine par "*Azotobacter chroococcum*." Ann. Inst. Pasteur, Paris, **122**: 1009–1028.

MULDER, E. G., and S. BROTONEGORO. 1974. Free-living heterotrophic nitrogen-fixing bacteria. *In* The biology of nitrogen fixation. *Edited by* A. Quispel. North-Holland Publishing Company, Amsterdam. pp. 37–85.

NEAL, J. L., and R. I. LARSON. 1976. Acetylene reduction by bacteria isolated from the rhizosphere of wheat. Soil Biol. Biochem. **8**: 151–155.

NELSON, A. D., L. E. BARBER, J. TJEPKEMA, S. A. RUSSEL, R. POWELSON, H. J. EVANS, and R. J. SIEDLER. 1976. Nitrogen fixation associated with grasses in Oregon. Can. J. Microbiol. **22**: 523–530.

NEYRA, C. A., J. DÖBEREINER, R. LALANDE, and R. KNOWLES. 1977. Denitrification by N_2-fixing *Spirillum lipoferum*. Can. J. Microbiol. **23**: 300–305.

OKON, Y., S. L. ALBRECHT, and R. H. BURRIS. 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl. Environ. Microbiol. **33**: 86–88.

PALLERONI, N. J., and A. V. PALLERONI. 1978. *Alcaligenes latus*, a new species of hydrogen-utilizing bacteria. Int. J. Syst. Bacteriol. **28**: 416–424.

PAUL, E. A., and J. D. NEWTON. 1961. Studies of aerobic non-symbiotic nitrogen-fixing bacteria. Can. J. Microbiol. **7**: 7–13.

POSTGATE, J. 1981. Microbiology of the free-living nitrogen-fixing bacteria, excluding *Cyanobacteria*. *In* Current perspectives in nitrogen fixation. *Edited by* A. H. Gibson and W. E. Newton. Australian Academy of Science, Canberra. pp. 217–228.

PRABHA, S., S. K. NAIR, K. R. DADARVAL, and P. TAURO. 1978. *Pseudomonas azotogensis*, as associate symbiont of Bajra (*Pennisetum americanum*). Plant Soil, **49**: 657–659.

PRIBRAM, E. 1933. Klassification der Schizomyceten. F. Deuticke, Leipzig. pp. 1–143.

PROCTOR, M. H., and P. W. WILSON. 1958. Nitrogen fixation by gram-negative bacteria. Nature (London), **182**: 891.

——— 1959. Nitrogen fixation by *Achromobacter* spp., Arch. Microbiol. **32**: 254–260.

RENNIE, R. J. 1980. Dinitrogen-fixing bacteria: computer-assisted identification of soil isolates. Can. J. Microbiol. **26**: 1275–1283.

——— 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. **27**: 8–14.

REYNDERS, L., and K. VLASSAK. 1979. Conversion of tryptophan acid by *Azospirillum brasilense*. Soil Biol. Biochem. **11**: 547–548.

RUBENCHIK, L. I. 1963. *Azotobacter* and its use in agriculture. (Translated from Russian.) Israel Program for Scientific Translations, Oldbourne Press, London.

SALKINOJA-SALONEN, M. S., E. VAISANEN, and A. PATERSON. 1979. Involvement of plasmids in the bacterial degradations of lignin-derived compounds. *In* Plasmids of medical, environmental and commercial importance. *Edited by* K. N. Timmis and A. Pühler. Elsevier/North-Holland, Biomedical Press, Amsterdam. pp. 301–314.

SINGH, M., and W. WENZEL. 1982. Detection and characterization of plasmids in *Azospirillum*. *In* Azospirillum, genetics, physiology, and ecology. *Edited by* W. Klingmüller. Exper. Suppl. (Base) **42**: 44–51.

SMYK, B., and L. ETTLINGER. 1963. Recherches sur quelques espèces d'*Arthrobacter* fixatrices d'azote isolées des roches karstiques alpines. Ann. Inst. Pasteur, Paris, **105**: 341–348.

TARRAND, J. J., N. R. KRIEG, and J. DÖBEREINER. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. **24**: 967–980.

THOMAS-BAUZON, D., P. WEINHARD, P. VILLECOURT, and J. BALANDREAU. 1982. The spermosphere model. I. Its use in growing, counting and isolating N_2-fixing bacteria from the rhizosphere of rice. Can. J. Microbiol. **28**: 922–928.

THOMPSON, J. P., and V. B. D. SKERMAN, 1979. *Azotobacteraceae*: the taxonomy and ecology of the aerobic nitrogen-fixing bacteria. Academic Press Inc., New York. 417.

TIEN, T. M., H. G. DIEM, M. H. GASKINS, and D. H. HUBBELL. 1981. Polygalacturonic acid transeliminase production by *Azospirillum* species. Can. J. Microbiol. **27**: 426–431.

VILLEMIN, G., J. BALANDREAU, and Y. DOMMERGUES. 1974. Utilisation du test de réduction de l'acétylène pour la numération des bactéries libres fixatrices d'azote. Ann. Microbiol. **24**: 87–94.

VOETS, J. P., and J. DEBACHER. 1956. *Pseudomonas azotogensis* nov. spp. A new free-living nitrogen-fixing bacterium. Naturwissenschaften, **43**: 40–41.

WATANABE, I., and W. L. BARRAQUIO. 1979. Low levels of fixed nitrogen required for isolation of free-living N_2-fixing organisms from rice roots. Nature (London), **277**: 565–566.

WIEGEL, J., D. WILKE, J. BAUMGARTEN, R. OPITZ, and H. G. SCHLEGEL. 1978. Transfer of the nitrogen-fixing hydrogen bacterium *Corynebacterium autotrophicum* Baumgarten et al. to *Xanthobacter* gen. nov. Int. J. Syst. Bacteriol. **28**: 573–581.

WILKE, D. 1980. Conjugational gene transfer in *Xanthobacter autotrophicus* GZ29. J. Gen. Microbiol. **117**: 431–436.

WINOGRADSKY, S. 1893. Sur l'assimilation de l'azote gazeux de l'atmosphère par les microbes. C.R. Acad. Sci. Paris, **116**: 1385–1388.

——— 1938. Etudes sur la microbiologie du sol et des eaux. Sur la morphologie et l'oecologie des *Azotobacter*. Ann. Inst. Pasteur, Paris, **60**: 351–400.

WOOD, A. G., E. M. MENEZES, C. DIKSTRA, and D. E. DUGGAN. 1982. Methods to demonstrate the megaplasmids (or minichromosomes) in *Azospirillum*. *In Azospirillum*, genetics, physiology, and ecology. Exper. Suppl. (Basel), **42**: 18–34.

YOSHIDA, T. 1970. Atmospheric nitrogen fixation. Annual report. International Rice Research Institute, Manila, Philippines. pp. 47–70.

YOSHIDA, T., and M. SUZUKI. 1975. Formation and degradation of ethylene in submerged rice soils. Soil Sci. Plant Nutr. (Tokyo), **21**: 129–135.

ZUBERER, D. A., and M. ROTH. 1982. *In vitro* inhibition of nonsymbiotic nitrogen-fixing bacteria by rhizosphere actinomycetes associated with grasses. Can. J. Microbiol. **28**: 705–709.